Dystroglycan and Protein O-mannosyltransferases 1 and 2 are Required to Maintain Integrity of Drosophila Larval Muscles

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ABSTRACT

In vertebrates, mutations in Protein O-mannosyltransferase1 (POMT1) or POMT2 are associated with muscular dystrophy due to a requirement for O-linked mannose glycans on the Dystroglycan (Dg) protein. In this study we examine larval body wall muscles of Drosophila mutant for Dg, or RNAi knockdown for Dg, and find defects in muscle attachment, altered muscle contraction and a change in muscle membrane resistance. To determine if POMTs are required for Dg function in Drosophila we examine larvae mutant for genes encoding POMT1 or POMT2. Larvae mutant for either POMT, or doubly mutant for both, show muscle attachment and muscle contraction phenotypes identical to those associated with reduced Dg function, consistent with a requirement for O-linked mannose on Drosophila Dg. Together these data establish a central role for Dg in maintaining integrity in Drosophila larval muscles and demonstrate the importance of glycosylation to Dg function in Drosophila. This study opens the possibility of using Drosophila to investigate muscular dystrophy.
INTRODUCTION

Dystroglycan (Dg) is an extracellular matrix (ECM) receptor for a variety of ligands and it plays important roles in epithelial polarity, muscle viability, neural migration and cancer progression (Winder, 2001). In skeletal muscle, as part of the Dystrophin Glycoprotein Complex (DGC), Dg spans the sarcolemma and provides a link between the ECM and the cytoskeleton. Disruption of this complex leads to muscle dystrophy (Blake et al., 2002; Deconinck and Dan, 2007). Dg protein is heavily glycosylated in its extracellular mucin-like domain with a variety of glycans, including O-linked mannose and mucin-type O-GalNAc structures (Ervasti and Campbell, 1993; Michele and Campbell, 2003; Combs and Ervasti, 2005). Mutations in glycosyltransferases that are involved in the post-translational modification of Dg are likewise associated with muscular dystrophy and neural migration defects (Muntoni et al., 2004; Kanagawa and Toda, 2006). These phenotypes seem directly due to the impact of these mutations on Dg and reflect the importance of glycosylation and post-translational processing to Dg function.

The O-mannose glycans present on Dg have received particular attention as these play a role in ligand binding. In particular, Dg lacking O-mannose glycans show reduced binding to laminin (Lam) (Chiba et al., 1997; Michele et al., 2002; Michele and Campbell, 2003; Muntoni et al., 2004). The initial step in generating these glycans is the addition of mannose in an O-linkage onto Serine or Threonine residues of Dg. Biochemical data indicates that this activity is carried out by two proteins, POMT1 and POMT2, which together form a biochemically active complex. Increased protein O-mannosylation in human cultured cells is only observed when both enzymes are
transfected together (Manya et al., 2004) and a further study demonstrated physical association between the two enzymes (Akasaka-Manya et al., 2006). Genetic data demonstrates a requirement for both POMT1 and POMT2, as mutation of either protein results in muscular dystrophy and associated neural defects in humans and other mammals (Akasaka-Manya et al., 2004; van Reeuwijk et al., 2005).

Genetic studies in model invertebrates, where genes are often present in single copy and genetic studies are less complicated by redundancy, have proven useful for furthering our understanding of cellular mechanisms involved in complex disorders. The main components of the mammalian DGC are conserved in *Drosophila* (Greener and Roberts, 2000; Neuman et al., 2001). Studies have identified a roles for Dg in epithelial and oocyte polarity (Deng et al., 2003; Poulton and Deng, 2006; Schneider et al., 2006) and recently age-dependent changes in adult muscles with reduced Dg levels have been identified (Shcherbata et al., 2007). Interestingly, several *Drosophila* Dg isoforms are generated via alternative splicing (Deng et al., 2003; Schneider et al., 2006). Only one of these contains the full mucin-like domain and it appears to be subjected to significant levels of glycosylation (Schneider et al., 2006). Dg isoforms that lack the mucin-like domain are required to maintain polarity in the follicular epithelium (Schneider et al., 2006), suggesting that the Dg isoforms can have different functional roles in *Drosophila*.

POMTs are also found in *Drosophila* (Martin-Blanco and Garcia-Bellido, 1996; Ichimiya et al., 2004; Lyalin et al., 2006) and biochemical analysis of *Drosophila* POMT1, encoded by the gene *rotated abdomen (rt)* (Martin-Blanco and Garcia-Bellido, 1996) and POMT2, encoded by *twisted (tw)* (Lyalin et al., 2006) has demonstrate that these enzymes possess POMT activity similar to their vertebrate homologs (Ichimiya et al., 2004). When transfected together in insect cells they are able to transfer mannose to a
GST-α-Dg substrate generating O-linked mannose (Ichimiya et al., 2004). In addition, reducing the level of either enzyme in vivo by RNAi resulted in reduced POMT activity of larval extracts towards the Dg substrate (Ichimiya et al., 2004). Mutation of the Drosophila enzymes or reducing their function using RNAi results in rotation of adult abdominal muscles, suggesting the enzymes have some role in muscle function or development (Martin-Blanco and Garcia-Bellido, 1996; Ichimiya et al., 2004; Lyalin et al., 2006). However, it is unclear if these phenotypes are related to a role for the enzymes in Dg modification and function as such a phenotype has not been described in Dg mutants.

In this study we examined Drosophila larval body wall muscles and identified errors in muscle attachment, changes in muscle contraction, and reduced cellular integrity associated with Dg mutant alleles and RNAi mediated reduction of Dg. We also find similar defects in larvae mutant for rt or tw or double mutant for both, and show genetic interactions between rt, tw and Dg, suggesting that the glycosyltransferases function in the same molecular pathway as Dg. These data are consistent with a non-redundant requirement for POMT enzymes, and the O-linked mannose glycans they generate, for proper Dg function in Drosophila.
MATERIALS AND METHODS

Drosophila Stocks and Crosses: \( Dg^{248}, Dg^{323} \) are previously described P-element excision alleles, \( Dg^{248} \) results in reduced Dg expression while \( Dg^{323} \) is null (Deng et al., 2003; Shcherbata et al., 2007). UAS-Dg-i, UAS-Dg-C, UAS-Dg-cyt have been previously described (Deng et al., 2003). \( rt^2 \) and \( rt^p \) are semi-lethal P-element insertion alleles as described (Martin-Blanco and Garcia-Bellido, 1996). \( tw^1 \) is viable and carries an amino acid substitution in the conserved POM domain (Lyalin et al., 2006). Lethal and semi-lethal alleles were balanced over GFP marked balancers, absence of GFP was used to select homozygous mutant larvae. Double mutant stocks were also generated with GFP marked balances (\( tw^1/FM7 \) GFP; \( rt^1/TM3 \) GFP, \( tw^1/FM7 \) GFP; \( rt^p/TM3 \) GFP, \( tw^1/FM7 \) GFP; \( Dg^{323}/CyO \) GFP, \( Dg^{323}/CyO \) GFP; \( rt^1/TM3 \) GFP). Gal4 drivers used: 24B-Gal4, 69B-Gal4, P-tub-Gal4. All crosses were maintained at 25°C. For 30°C experiments crosses were set up at 25°C and adults allowed to lay eggs for two days, vials were then transferred to 30°C.

Muscle Measurements: Larvae were dissected, fixed and stained with phalloidin; muscles were imaged and measurements obtained as described previously. All muscle measurements were from muscles 6/7 abdominal segments 3 or 4. Sarcomere measurements were from the central region of muscle 6 abdominal segments 3 or 4. No more than two muscles were measured from a single larvae. Sarcomeres were measured from grey scale intensity plots across phalloidin stained sarcomeres (fig. 2 and 3 and (Haines and Stewart, 2007), sarcomere size being the distance between adjacent peaks. In
cases where the graph did not clearly show a single peak for a sarcomere, the center of
the high intensity region was selected by eye as the peak value.

Surface area of the third instar larval muscles 6/7 from which sarcomere measurements
were taken were: (mean±SEM μm², N = number muscles): Figure 3b 25°C yw
76334±2191, 22; UAS-Dg-i/+ 76674±2281, 21; 24B-Gal4 /+ 74794±1579, 22; P-tub-
Gal4/+ 74398±3260, 18; 24B-Gal4::UAS-Dg-i 72544±2006, 22; P-tub-Gal4::UAS-Dg-i
71404±2677, 14; 69B-Gal4::UAS-Dg-i 79663±2124, 12. Figure 4b: 30°C yw
71010±2149, 24; UAS-Dg-i/+ 77368±1554, 20; 24B-Gal4/+ 80932±1478, 16; 24B-
Gal4::UAS-Dg-i 76610±1973, 26. Figure 7f: tw¹ 77698±1789, 50; rt² 64747±1926, 19;
rt²/rt⁰ 70809±3759, 22; tw¹;rt² 60834±1884, 29; tw¹; rt²/rt⁰ 62877±1725, 17.

**Antibody Staining:** Third instar larvae were dissected along the dorsal midline in zero
calcium HL3 saline (Stewart and McLean, 2004). Upon removal of the digestive tract, fat
bodies and main trachea, the preparations were fixed in 4% formaldehyde in phosphate
buffered saline (PBS) for 10 minutes. The tissue was then washed in PBS, 0.1% triton,
0.5% BSA (PBT) and stained in PBT + 5% normal goat serum, overnight a 4°C or for
anti-Dlg and anti-HRP at room temperature for 1 hour. Antibodies used were: rabbit anti-
Dg (1:800) (Deng *et al.*, 2003), guinea pig anti-Lam (1:1000) (gift T. Volk) rabbit anti-
Dys (1:1000) (Schneider *et al.*, 2006), mouse anti-Dlg (1:200)(Developmental Studies
Hybridoma Bank (DSHB)), Texas Red goat anti-HRP (Jackson ImmunoResearch) at
1:100, rat anti-alpha actinin (Babraham Bioscience Technologies) 1:10. Secondary
antibodies were made in goat and purchased from Molecular Probes; anti-rabbit
Alexafluor488, anti-guinea pig Alexafluor633, anti-mouse Alexafluor633, anti-rat Alexafluor488. Antibody staining was, where appropriate, followed by lectin (see below) or phalloidin staining. Tetramethylrhodamine isothiocyanate-Phalloidin (Sigma) or FITC-Phalloidin (Sigma) was used at 1μg/ml for 30 minutes.

Stained preparations were mounted in 90% glycerol in PBS, or where indicated Vectashield with propidium iodide (Vector Labs). Images of antibody stained muscle sarcomeres were collected on a Zeiss LSM 510 confocal microscope using F-Flura 40X/1.3 oil lens with 2X zoom. The pinhole was set to 70μm, with a resulting confocal section thickness of 0.6μm. Confocal images were exported to imageJ (NIH). Low magnification images of whole muscles stained with antibodies or with phalloidin were taken with a 10X lens on a Nikon E600FN microscope equipped with a Hamamatsu Orca ER CCD camera. Images then were obtained from the camera with SimplePCI (Compix Inc.) software and exported to imageJ. Where comparisons were made (fig. 4c, 5a, 6a and b, 6c and d) samples were stained in the same tube, mounted together on a slide and imaged with the same settings.

**Lectin staining:** Larvae were dissected, fixed and washed as for antibody staining and incubated with 4μg/ml lectin in PBT for 30 minutes at room temperature. Lectins used were: Fluorescein-VVA, Rhodamine-SJA, Fluorescein-GSL-II, Rhodamine-WGA, Fluorescein-SNL, all from Vector Labs.

**Western blot:** Six 3rd instar larvae of each genotype were dissected along the dorsal midline in zero calcium HL3 saline. Digestive tract, fat bodies, main trachea and CNS were removed. Tissue was transferred to 12μl of buffer containing 50mM Tris pH 7.4,
1% triton with complete proteases inhibitors (Roche). The sample was homogenized with a micro tissue grinder and incubated at 4°C for 20 minutes. 12μl 2X SDS loading buffer +DDT was added and samples were boiled for 5 minutes. 15μl of each sample was separated on a 6% SDS-polyacrylamide gel and western transferred to PVDF membrane. The membrane was incubated with rabbit anti-Dg (1:3000) and detected with an HRP-coupled secondary antibody, followed by chemiluminescent detection (ECL Amersham). 4μl of each sample was loaded on 10% SDS-polyacrylamide gel, blotted and incubated with β-tubulin (1:100 MabE7, Developmental Studies Hybridoma Bank) for loading control.

**Physiology:** Physiology assays were carried out in HL3 saline (Stewart *et al.*, 1994) with 0.75mM calcium, as previously described (Stewart and McLean, 2004). EJPs were analyzed using the cursor options of Clampfit 10.0, mEJPs were analyzed with the template search option. Sample size was 7 for each genotype.

**Statistics:** One-way ANOVA tests were used for statistical comparisons except where indicated. Tests were carried with Graphpad Prism 4.0 software, with P<0.05 chosen as the level of significance.
RESULTS

**Muscle attachment defects in Dystroglycan mutants**

To determine if $Dg$ plays a role in *Drosophila* muscles we examined the musculature of larvae carrying mutant alleles of $Dg$. $Dg$ alleles have previously been generated and described (Deng *et al.*, 2003; Shcherbata *et al.*, 2007); we examined larvae homozygous for a null allele, $Dg^{323}$, a hypomorph, $Dg^{248}$ and $Dg^{323}/Dg^{248}$ larvae. These $Dg$ mutants survive until early third instar; we dissected late second instar larvae to reveal the neuromuscular system and stained with fluorescently labeled phalloidin to visualize the muscles. In wild-type larvae, muscles are arranged in a stereotypical pattern and are attached to the underlying epidermis via a link with tendon cells at defined locations (Bate, 1990; Prokop *et al.*, 1998; Volk, 1999) (fig. 1a). In abdominal segments 2-7, 30 individual muscles are present in each hemisegment. We found that in $Dg^{323}$, $Dg^{248}$ or $Dg^{323}/Dg^{248}$ larvae one or more muscles were frequently missing or mis-attached (fig. 1b-f and table 1). In some larvae a single muscle from one hemisegment was affected, whilst in other cases more than one hemisegment was affected. No consistent pattern as to which hemisegment or muscle was affected could be determined. Occasionally additional muscle tissue was present (fig. 1c). Some examples of muscle mis-attachment occurred in hemisegments that lacked a muscle and may result from rearrangement of the remaining muscles. In other examples, mis-attachment was observed despite a normal number of muscles in the hemisegment. Together these results demonstrate that $Dg$ plays a role in normal muscle attachment in *Drosophila*. 
Dystroglycan mutant muscles have altered sarcomere size

In the course of examining the muscle attachment phenotypes we also noticed that some muscles appeared to be shorter and wider than normal (see below). To examine the underlying muscle sarcomeres we stained the muscles with phalloidin which labels the F-actin present in the muscle contractile machinery resulting in broad stripes along the muscles (fig. 2). The center of each F-actin band, the Z-band, stains more intensely with phalloidin. The spacing of the F-actin bands can be analyzed by graphing gray scale intensity across phalloidin stained sarcomeres, Z-bands are represented as peaks and the distance between peaks can be used as a measure of sarcomere size and the width of the curve used to assess the width of the F-actin band (fig. 2)(Haines and Stewart, 2007). We examined the spacing of the F-actin bands along muscle 6, segments 3 or 4, when the banding pattern across the entire length of the muscle was intact (yw N=8, Dg\textsuperscript{248} N=8, Dg\textsuperscript{323}/Dg\textsuperscript{248} N=8, Dg\textsuperscript{323} N=6).

Firstly, we found that yw larvae showed very consistent sarcomere sizes of approximately 8 μm (8.01±0.07 μm, N=40 sarcomeres from 8 muscle) (fig. 2d). In contrast, Dg\textsuperscript{248} mutants the F-actin bands appeared closer together than in controls; on average Dg\textsuperscript{248} sarcomeres were 7.3 ± 0.07 μm (N=53 sarcomeres from 8 muscles), but the width of the F-actin band was unaffected (fig. 2b, d). This could suggest the muscles are hypercontracted. We would expect hypercontracted muscles to be wider and shorter than controls and so measured the width and length of the muscles, together with overall muscle surface area. The width of muscle 6/7 combined from Dg\textsuperscript{248} mutants was larger than controls, whilst muscle length was smaller (width±SEMμm, length±SEMμm, N muscles; yw 61.3±1.2, 195.2±6.3, 41; Dg\textsuperscript{248} 66.8±1.6, 166.8±2.6, 27). This resulted in a significantly different width/length ratios for the Dg\textsuperscript{248} mutants compared to controls.
(P<0.001) (width/length±SEM, N muscles; yw 0.33±0.011, 41; Dg^{248} 0.41±0.014, 27). No significant difference in surface area was found (P>0.05) (area±SEMμm^2, N muscles; yw 12844±577, 41; Dg^{248} 11566±239, 27). This change in muscle shape, but no change in surface area is consistent with the Dg^{248} muscles being hypercontracted.

Further examination of the Dg^{248} data revealed that, along with the reduction in the mean value, the sarcomere measurements were more variable. For example, the coefficient of variation (standard deviation / mean) was 5.6% for yw samples compared to 7.8% for Dg^{248} samples. This greater variability is also evident from the scatter plot shown in Figure 2d.

We next examined stronger Dg combinations, including Dg^{323}/Dg^{248} and Dg^{323} mutants. The mean value for all the data from Dg^{323}/Dg^{248} muscles was similar to yw: 8.26±0.15 μm, N=39 sarcomeres from 8 muscles. However the data was obviously more variable than in the yw sample with a coefficient of variation of 12%, and 14 sarcomeres larger than the largest measured from the yw sample. For Dg^{323}, the mean value for all the data was increased to 8.7±0.12μm, N=35 sarcomeres from 6 muscles and the coefficient of variation was 8.4%. In Dg^{323} samples there were 18 sarcomeres larger than the largest yw sarcomere.

Analysis of variance was performed on the sarcomere size data from the yw, Dg^{248}, Dg^{323}/Dg^{248} and Dg^{323} groups to test for differences among the mean values. This revealed significant variation between the groups (P<0.0001). Post tests revealed that only yw compared to Dg^{323}/Dg^{248} was not different (P>0.05), whereas all other combinations were different from each other (P<0.01). Furthermore, Bartlett’s test for equal variance revealed a significant (P<0.001) difference in the sample variability between the genotypes.
Together these results show $Dg$ alleles can significantly affect sarcomere size. The weakest allele causes sarcomeres to become smaller while increasing allelic strength results in progressively larger sarcomeres, but also a higher degree of variability in the mutant lines. Since $Dg^{323}$ is a null allele these data indicate the strongest loss of function phenotype is longer sarcomeres.

**RNAi knockdown of Dg alters sarcomere size**

To overcome the lethality associated with the $Dg$ mutant alleles and to investigate the requirement for $Dg$ in different tissues we turned to an RNAi approach. We expressed a UAS coupled RNAi construct against $Dg$ with a mesoderm driver (24B-Gal4), a ubiquitous driver (P-\textit{tub}-Gal4) and a pan-ectodermal driver (69B-Gal4). While 24B-Gal4 UAS-$Dg$-i was viable, some lethality during larval stages was observed with 69B-Gal4 and P-\textit{tub}-Gal4, however in each case adult flies could be obtained.

We examined third instar larval muscles to determine if they showed a change in sarcomere size similar to those we had observed in the $Dg$ mutants. Driving expression of the UAS-$Dg$-i construct with P-\textit{tub}-Gal4 resulted in sarcomeres that were significantly smaller than controls (P<0.001) (fig. 3a, b). With 24B-Gal4 we observed a more variable phenotype. Some muscles had small sarcomeres and others were large. Generally, individual larvae showed either small or large sarcomeres in each of its muscles. We did not observe both small and large sarcomeres in a single muscle. This variable phenotype resulted in the 24B-Gal4:UAS-$Dg$-i having a similar mean sarcomere size as controls but a larger variation (fig. 3b). This increased variation resulted in a coefficient of variation value of 14.35% for 24B-Gal4:UAS-$Dg$-i compared to 7.03% for $yw$ and 5.21% and 6.21% for UAS-$Dg$-i/+ and 24B-Gal4/+ respectively. Furthermore, Bartlett’s test for
equal variance revealed a significant (P<0.001) difference in the sample variability between the genotypes.

Expressing UAS-Dg-i with 69B-Gal4 did not affect sarcomeres size (fig. 3b). We did not observed a difference between the overall size of the muscles across the different genotypes as measured by surface area of muscle 6 and 7 combined (P>0.05). Together these results demonstrate that loss of Dg in muscle results in altered sarcomere size in third instar larval muscles, and that both smaller and larger sarcomeres can result from reduced Dg levels. This is consistent with our analysis of the 2nd instar Dg mutant muscles where we observed examples of smaller and larger sarcomeres. In the Dg mutants we had found that the hypercontraction phenotype was predominant amongst larvae homozygous for the Dg hypomorphic allele Dg^{248}, whilst wider, more relaxed sarcomeres was more common in Dg^{123}/Dg^{248} or homozygous null Dg^{323} individuals. This suggests that more severe loss of Dg may tend to result in larger sarcomeres and weaker Dg knockdown could result in hypercontraction.

To test this idea we took advantage of the temperature dependency of the Gal4 system and enabled the 24B-Gal4::UAS-Dg-i larvae to develop at 30°C, rather than at 25°C as in our experiments described above. This resulted in a stronger decrease of Dg protein on western blots compared to 24B-Gal4::UAS-Dg-i larvae raised at 25°C (fig. 4a). Whereas the 24B-Gal4::UAS-Dg-i larvae raised at 25°C had shown a high degree of variance amongst sarcomeres the variance for the 24B-Gal4::UAS-Dg-i sarcomeres at 30°C was similar to controls (fig. 4b). The coefficient of variation value was 8.6% for 24B-Gal4::UAS-Dg-i raised at 30°C compared to 8.0% for yw, 6.5% for 24B-Gal4/+ and 5.2% for UAS-Dg-i/+ when raised at 30°C. Although no significant change in variation between the samples was observed the mean sarcomere size was significantly larger for
the 24B-Gal4::UAS-\textit{Dg}-i compared to the control groups (P<0.001)(fig 4b). Together these results indicate that strong 24B-Gal4 driven Dg RNAi knockdown results in sarcomeres that are consistently larger than controls (fig. 4b), and together with our results from RNAi knockdown experiments at 25°C suggests that the larger sarcomere phenotype is the result of a more severe loss of Dg compared to the variable or hypercontracted phenotype.

As with the smaller sarcomeres, we did not observe a difference in the width of the phalloidin stained band (fig. 4c). To further examine the 24B-Gal4::UAS-\textit{Dg}-i 30°C muscles we used an antibody against the Z-band marker alpha-actinin and found that this protein was localized normally. The apparently normal arrangement of F-actin and the Z-band maker in the 24B-Gal4::UAS-\textit{Dg}-i muscles suggests that despite the changes in sarcomere size the contractile machinery remains intact. Further the \textit{Dg} RNAi knockdown and \textit{Dg} mutant larvae appear to crawl normally suggesting that the ability of the muscles to contract is not significantly compromised. We conclude the simplest explanation for the change in sarcomere size we observe is altered muscle contraction.

The third instar UAS-\textit{Dg}-i larvae were also examined for muscle attachment defects however driving expression with 24B-Gal4, 69B-Gal4 or \textit{P-tub}-Gal4 did not result in a significant number of attachment defects when compared to the Gal4 drivers alone. This suggests that the temporal or spatial knockdown of Dg achieved with these drivers is insufficient to disrupt this aspect of \textit{Dg} function.

\textbf{Electrical properties of \textit{Dg} RNAi muscles}

In vertebrate muscles lacking components of the DGC membrane fragility leading to microlesions in the membrane have been suggested as early events that begin a cycle
of muscle damage (Deconinck and Dan, 2007). We used intracellular recording
techniques to measure membrane resistance, membrane potential and both spontaneous
(miniature excitatory junctional potentials (mEJPs)) and nerve-stimulated (excitatory
junctional potentials (EJPs)) synaptic release to determine if the muscle membrane
function or synaptic function was compromised in the 24B-Gal4::UAS-Dg-i muscles. We
found that the resting membrane potential was similar for all genotypes (Table 2) but that
there was a significant difference in membrane resistance between the controls (UAS-Dg-i/+)
and 24B-Gal4::UAS-Dg-i larvae that were raised at either 25°C and at 30°C (P<0.05
for both comparison; Table 2). However, rather than decreased membrane resistance as
we expected if membrane were more permeable due to lesions, we found a significantly
higher resistance in 24B-Gal4::UAS-Dg-i muscles. In line with the increased membrane
resistance, the EJP amplitude was also significantly increased. No change in mEJP
amplitude was detected, although the mean value was increased in 24B-Gal4::UAS-Dg-i
muscles at both temperatures. Together these results indicate a change in membrane
function in the 24B-Gal4::UAS-Dg-i muscles compared to controls.

Dystroglycan is associated with the T-tubule network in larval muscles

Dg expression has previously been reported in muscle fibers in Drosophila
embryos by in-situ hybridization (Dekkers et al., 2004), however, its sub-cellular location
has not been reported. As we have identified phenotypes when Dg levels are reduced in
muscles with RNAi we sought to establish the sub-cellular localization of Dg in the
Drosophila larval muscles using an anti-Dg antibody (Deng et al., 2003). Drosophila Dg
seems not to be cleaved into α and β sub-units but remains as a single polypeptide (Deng
et al., 2003) and this antibody recognizes an epitope in the intracellular domain (Deng et
al., 2003). Dg staining was observed in puncta on the top of the muscle cells (fig. 5a, b). In the internal area of the muscles Dg staining was present in repeating stripes through the sarcomeres (fig. 5d). Given the transmembrane domain in Dg we reasoned that this could represent Dg at the transverse (T)-tubule membrane. These invaginations of the plasma membrane penetrate deep into the muscle and are involved in excitation contraction coupling. In vertebrate cardiac muscle Dg, its associated protein Dystrophin (Dys), and Lam are found in regions where T-tubules are located (Klietsch et al., 1993; Kaprielian et al., 2000) and the staining pattern of Dys in Drosophila larval muscles has been suggested to be T-tubule associated (van der Plas et al., 2006). We stained muscles with an antibody against the known T-tubules marker Discs Large (Dlg) (Razzaq et al., 2001) and found that the Dlg and Dg signals are localized in a similar pattern across the sarcomeres (fig. 5c-f). The similar pattern but the lack of specific colocalization suggests that these proteins are localized to different regions of the T-tubule network.

**N-terminal Dystroglycan extends into the T-tubule lumen**

If Dg is associated with the T-tubule membrane we would expect that its N-terminal domain would extend into the T-tubule lumen, allowing Dg to interact with extracellular ligands. As vertebrate α-Dg is heavily glycosylated in its mucin domain we investigated the possibility that lectins could be used to specifically recognize the extracellular region of Dg in Drosophila muscles via glycan structures. Therefore, we first needed to validate potential lectins as tools to probe Dg localization.

To identify potential Dg binding lectins we overexpressed a UAS-Dg (Deng et al., 2003) construct using 24B-Gal4; this full-length construct encodes the Dg-C isoform that contains the entire mucin domain (Deng et al., 2003; Schneider et al., 2006). In this
overexpression situation, anti-Dg antibody reveals high levels of Dg present in the sarcomeres and strong accumulation at the post-synaptic side of the neuromuscular junction (fig. 6a). Therefore, lectins that bind Dg should also show ectopic post-synaptic staining in the UAS-Dg muscles. No change in staining pattern was observed with wheat germ agglutinin (WGA), *Sophora Japonica* agglutinin (SJA), or *Griffonia Simplicifolia* lectin II (GSL-II) (data not shown), however background staining may interfere with our ability to detect an increase in staining at the NMJ with these lectins. *Sambucus Nigra* lectin (SNA), that has specificity towards sialic acid, showed some increase in post-synaptic staining, but *Vicia Villosa* Agglutinin (VVA), that binds *N*-acetylglactosamine-terminated glycoconjugates, showed a dramatic accumulation at post-synaptic sites in 24B-Gal4::UAS-Dg-C muscles, but no significant post-synaptic staining in yw controls (fig. 6a and data not shown, compare to fig. 6b).

To confirm that VVA was binding to the N-terminal region of Dg we expressed a truncated *Dg* construct that lacks the entire extracellular domain, UAS-Dg-cyt (Deng et al., 2003). The Dg antibody recognized this truncated protein and the antibody showed strong staining in the sarcomeres and in post-synaptic regions, similar to overexpression of the full-length construct (fig. 6b), however no VVA staining was detected at post-synaptic regions in these muscles. These results demonstrate that VVA can recognize the extracellular domain of Dg.

We next examined VVA staining in wild type muscles and observed staining in broad stripes in the sarcomeres overlapping the phalloidin signal (fig. 6c). This staining is reduced in 24B-Gal4::UAS-Dg-i 30°C muscles (fig. 6d). Altogether these results demonstrate that Dg is a major VVA reactive protein in the larval muscles, and suggest that *Drosophila* Dg is modified with GalNAc terminated glycans. The VVA staining
pattern establishes the location of N-terminal Dg in broad stripes that appear to overlap phalloidin. This staining pattern likely represents N-terminal Dg projecting into the T-tubule lumen.

**Laminin and Dystrophin are T-tubule associated**

The N-terminal region of Dg interacts with a number of extracellular ligands and in muscles a major ligand is Lam. We sought to establish if Dg could potentially interact with Lam in *Drosophila* larval muscles. Using an antibody against Lam we found that this protein is present in repeating stripes through the sarcomeres where it overlaps with the VVA signal (fig. 6c).

In vertebrates binding between Dg and Lam provides a link between the membrane and the extracellular matrix (Blake *et al.*, 2002); a second link between the intracellular C-terminal domain of Dg and the actin binding protein Dys serves to anchor the complex to the cytoskeleton (Blake *et al.*, 2002). A previous study demonstrated that Dys is found in repeating stripes in the *Drosophila* larval muscles and suggested the possibility that Dys could be associated with T-tubules (van der Plas *et al.*, 2006). Altogether the location and proximity of the core DGC proteins, Dg and Dys, and the presence of Lam in the *Drosophila* muscles indicated that a T-tubule DGC-like complex is present in the larval muscles.

**Mutants in POMTs display muscle attachment defects and altered sarcomere size**

Having established that Dg is required for normal muscle integrity and provided evidence that Dg is localized to T-tubules, along with the other known members of the DGC complex, we next examined the importance of glycosylation to Dg function.
Mutations in POMT1 or POMT2 are associated with muscular dystrophy in vertebrates due to a requirement for O-linked mannose glycans on the Dg protein (Akasaka-Manya et al., 2004; van Reeuwijk et al., 2005). We investigated whether POMTs may be required for Dg function in Drosophila by examining larvae mutant for either rt (POMT1) or tw (POMT2) or doubly mutant for both. Muscle attachment defects have previously been reported in rt mutants (Martin-Blanco and Garcia-Bellido, 1996) and our analysis found muscle attachment errors in single rt and tw mutants and in double mutants (fig. 7a-e). These defects occurred at a similar frequency to those seen in the Dg mutants (table 3). Examples of muscle absence, mis-attachment and extra or split muscle fibers were observed (fig. 7a-e). To determine if these mutants showed changes in muscle contraction sarcomere size was measured from third instar larval muscles. tw and rt mutants and tw;rt double mutant sarcomeres were all larger than controls (fig. 7f) and were similar in size to the sarcomeres of 24B-Gal4::UAS-Dg-i larvae that had been raised at 30°C. Together this data is consistent with a requirement for O-linked mannose glycans on Drosophila Dg and supports biochemical data that indicates a non-redundant role for these enzymes in modifying Dg (Ichimiya et al., 2004).

**Genetic interactions between POMTs and Dystroglycan**

To further establish a link between POMTs and Dg we generated larvae that carried mutations in tw or rt in combination with Dg alleles and examined the musculature for the attachment and sarcomere size phenotypes. Both tw and rt interacted with Dg. tw/++; Dg^{248}/+ third instar larvae showed muscle attachment defects (0.110/hemisegment, N=82), but normal sized sarcomeres (8.34±0.09μm N=73). A strong interaction was observed between Dg and rt, the Dg^{323}/++; rt^{p}/+ combination was
semi-lethal, larvae that survived to third instar showed muscle attachment defects (0.073/hemisegment, N=82) and large sarcomeres (9.045±0.07μm N=47). These interactions support a role for the glycosyltransferases in the same molecular pathway as Dg.

We next examined double mutant combinations. We found that tw/Y;Dg<sup>323</sup>/Dg<sup>323</sup> or Dg<sup>323</sup>/Dg<sup>323</sup>; rt<sup>2</sup>/rt<sup>2</sup> animals showed an identical phenotype to the Dg<sup>323</sup>/Dg<sup>323</sup> single mutants. The larvae survived until early 3<sup>rd</sup> instar and tw/Y;Dg<sup>323</sup>/Dg<sup>323</sup> (N=4) and Dg<sup>323</sup>/Dg<sup>323</sup>; rt<sup>2</sup>/rt<sup>2</sup> (N=6) larvae showed changes in sarcomere size and muscle attachment defects. Attachment defects were observed at a frequency of 0.094/hemisegment, N = 32 for tw/Y;Dg<sup>323</sup>/Dg<sup>323</sup> and at 0.091/hemisegment, N = 33 for Dg<sup>323</sup>/Dg<sup>323</sup>; rt<sup>2</sup>/rt<sup>2</sup>. The lack of additional phenotypes in these double mutant combinations, compared to the Dg<sup>323</sup>/Dg<sup>323</sup> single mutants, argues that Dg is the major functional substrate for these glycosyltransferases in Drosophila.
DISCUSSION

The DGC is known to play a central role in maintaining integrity in a variety of different muscle types. In this study we established that Dg is required in *Drosophila* larval muscles to maintain integrity. We have identified changes in muscle contraction associated with reduced Dg function. Second instar $Dg^{248}$ larvae had short and wide muscles, and a decrease in sarcomere size, together indicating that the muscles are hypercontracted (fig. 2). Amongst larvae null for Dg we found that sarcomere size was more variable between individuals with some muscles having smaller sarcomeres and others larger (fig. 2). Amongst third instar larvae knockdown for Dg we also observed changes in sarcomere size. When a RNAi construct against Dg was driven with the ubiquitous driver P-*tub*-Gal4 we found that sarcomeres were consistently smaller than controls (fig. 3). Driving the RNAi construct with the mesoderm driver 24B-Gal4 resulted in a variable phenotype with both small and large sarcomere being observed. This phenotype shifted to exclusively larger sarcomeres when these larvae developed at 30oC, likely due to a greater decrease of Dg protein (fig. 3b and 4). As the large sarcomere phenotype was seen in null Dg larvae and with strong RNAi knockdown compared to smaller sarcomeres in the $Dg^{248}$ individuals and with weaker RNAi knockdown of Dg we conclude that the large sarcomere phenotype is related to more severe loss of Dg compared with hypercontraction.

As we observed changes in muscle sarcomere size with RNAi driven with the ubiquitous driver and the mesoderm driver but not with the pan-ectodermal driver we conclude that the sarcomere size changes are due to loss of Dg in muscles. Together our
results demonstrate that reduced levels of Dg alter muscle contraction and that Dg plays a role in *Drosophila* muscles.

Consistent with our findings is that both hypercontraction and overstretching of sarcomeres is seen in vertebrate dystrophic muscles before they progress to a more advanced degeneration phenotype (Cullen and Fulthorpe, 1975). A tendency to hypercontract is also associated with disruption of DGC proteins in *C. elegans* (Bessou et al., 1998; Grisoni et al., 2002). These findings suggest that contractile changes are a common early response to lack of Dg function in different types of muscle. Further studies will focus on understanding whether these changes in muscle contraction stem from developmental changes in the muscles or are the result of early changes associated with muscle degeneration.

In vertebrate muscles lacking DGC components, the mechanisms leading to muscle dystrophy remain unclear, however membrane fragility is likely involved (Blake et al., 2002; Deconinck and Dan, 2007). Mechanical stress from muscle contraction is thought to lead to membrane microlesions and compromised muscle membrane function. Our electrophysiological analysis showed an increase in membrane resistance and greater EJP amplitude in 24B-Gal4::UAS-\(\text{Dg-i}\) muscles. The increase in passive membrane resistance could be due to downregulation of the leakage channel activity by the muscle. The increased resistance would help to maintain excitation-contraction coupling. Interestingly, a similar increase in muscle membrane resistance has been reported in mice mutant for *dystrophin (mdx)* (De Luca et al., 1997).

In vertebrate cardiac muscle T-tubule associated Dys, Dg and Lam has been reported (Klietsch et al., 1993; Kaprielian et al., 2000). This location differs from the sarcolemma localized DGC found in vertebrate skeletal muscle (Blake et al., 2002). The
inherited muscular dystrophies are associated with dilated cardiomyopathy and differences in the degree of dysfunction between cardiac and skeletal muscle in individuals with muscular dystrophy has lead to the suggestion that the DGC may have alternative cellular roles in these muscle types (Lapidos et al., 2004; Goodwin and Muntoni, 2005). Our finding that Dg and Lam are T-tubule associated in Drosophila larval muscles suggests these Drosophila muscles may provide a good model for investigating the cellular function of T-tubule associated DGC. Studying the role of the DGC in different types of muscle will increase our overall understanding of how this complex functions at both a molecular and cell biological level.

Lam and adhesion molecules such as intergrins play important roles in muscle attachment in Drosophila (Brown, 1994; Yarnitzky and Volk, 1995; Prokop et al., 1998; Delon and Brown, 2007). Muscles detach from the epidermis and round up or stick non-specifically to other muscles or the ECM in Drosophila carrying mutant alleles of these genes (Brown, 1994; Prokop et al., 1998). Given the role of Dg in ECM adhesion in other cellular contexts (Winder, 2001) and the role of Lam in muscle attachment in Drosophila (Prokop et al., 1998) the muscle attachment phenotypes we have observed in the Dg mutants most likely result from weakening of the connection between muscle and epidermal tendon cell to which it connects or these cells and the ECM. Such a weakness could explain the random nature of the phenotypes. Failure of a muscle to maintain its connection with the tendon cell could result in loss of the muscle, or result in the muscle forming a link with another muscle or tendon resulting in a mis-attachment phenotype.

In vertebrates, Dg function is regulated by glycosylation (Michele and Campbell, 2003; Muntoni et al., 2004; Barresi and Campbell, 2006). We observed changes in sarcomere size and defects in muscle attachment in Drosophila mutant for genes
encoding POMT1 and POMT2. We also found that both these mutants interact with Dg mutant alleles in trans-heterozygous combinations. The similarity of the rt and tw muscle phenotypes to those associated with reduced Dg function and the interactions between mutant alleles of rt, tw and Dg provide strong evidence that rt and tw are required for Dg dependent processes in Drosophila. Given the O-mannosyltransferase activity of the rt and tw gene products towards Dg (Ichimiya et al., 2004) and the genetic evidence from vertebrates that loss of POMTs results in hypoglycosylation of Dg and subsequent disruption to Dg function these data provide compelling evidence supporting a functional requirement for O-mannose glycans on Dg in Drosophila. The adult abdomen in tw and rt mutant flies is rotated. We have not observed this phenotype in our experiments with Gal4 driven Dg RNAi knockdown. Possibly the abdominal rotation is due to loss of Dg function in these mutants and that the lack of this phenotype in the RNAi flies is due to insufficient knockdown of Dg. It also remains possible that rt and tw have another substrate and loss of glycan structures on this protein results in the rotated abdomen phenotype.

Altogether the phenotypes we have identified by manipulating Dg, tw and rt demonstrate that Dg plays a central role in maintaining cell integrity in the Drosophila larval muscles and that glycosylation of Dg is important to its function. This report therefore opens the possibility of using genetic analysis of the highly accessible neuromuscular system available in this model organism to analyze the mechanisms by which loss of DGC function leads to muscle dystrophy.
ACKNOWLEDGEMENTS

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REFERENCES


Table 1: Frequency of muscle attachment defects in 2\textsuperscript{nd} instar Dg mutants

<table>
<thead>
<tr>
<th>Muscle defect</th>
<th>yw</th>
<th>D\textsubscript{g} \textsuperscript{248}</th>
<th>D\textsubscript{g} \textsuperscript{248}/D\textsubscript{g} \textsuperscript{248}</th>
<th>D\textsubscript{g} \textsuperscript{233}</th>
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<td>M5 or M8</td>
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<td>2</td>
<td>0</td>
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Table 2: Membrane resistance is increased in *Dg* RNAi knockdown muscles

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<th>Membrane Potential (mV)</th>
<th>Membrane Resistance (mΩ)</th>
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<th>mEJP Amplitude (mV)</th>
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<td></td>
<td>*Dg-i/+</td>
<td>24B-Gal4:: <em>Dg-i</em></td>
<td>*Dg-i/+</td>
<td>24B-Gal4:: <em>Dg-i</em></td>
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<td>Membrane Potential (mV)</td>
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<td>-65 ± 2.0</td>
<td>-67 ± 1.4</td>
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<td>Membrane Resistance (mΩ)</td>
<td>5.8 ± 0.45</td>
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<td>mEJP Frequency (Hz)</td>
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<td>1.13 ± 0.14</td>
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<td>44.41 ± 2.5 *</td>
<td>20.29 ± 2.3</td>
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<td>Quantal Content</td>
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<td>46.46 ± 2.7</td>
<td>30.76 ± 5.1</td>
<td>44.11 ± 2.9</td>
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*denotes P<0.05 two-way ANOVA

each parameter was analyzed by two-way ANOVA (genotype x temperature)
Table 3: Frequency of muscle attachment defects in third instar *rt* and *tw* mutants

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<th>Muscle defect</th>
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<th><em>rt</em></th>
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<td>1</td>
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<td>10</td>
<td>1</td>
<td>5</td>
<td>4</td>
<td>8</td>
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<tr>
<td>M6, 7, 12, 13</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>0</td>
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<tr>
<td>Total defects</td>
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<td>9</td>
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<td>60</td>
<td>82</td>
<td>128</td>
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<tr>
<td>Defects/hemisegment</td>
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<td>0.128</td>
<td>0.133</td>
<td>0.110</td>
<td>0.132</td>
<td>0.116</td>
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**FIGURE LEGENDS**

**Figure 1: Dg mutants have defects in muscle attachment.** Phalloidin stained musculature of 2nd instar larvae. (A) yw, wild type arrangement of muscles in part of a hemisegment, muscles referred to in text are labeled by number according to standard nomenclature. (B-F) Examples of muscle attachment defects in Dg mutants. (B) $Dg^{248}$, muscle 5, normally located between the arrowheads, is missing. (C) $Dg^{323}/Dg^{248}$, muscle 5 is underneath, rather than above, muscle 4 as indicated by arrowhead. Extra muscle tissue is also present (arrow). (D) $Dg^{248}$, muscles 6 and 7 cross over and swap their normal attachment sites. Arrowhead on the left side of the panel indicates normal attachment site for muscle 6, the muscle at this site is attachment to the normal muscle 7 location on the right hand side of the panel (arrowhead on right). The muscle at the normal muscle 7 attachment site on the left (arrow) crosses under the other muscle to attach on the right-hand side, as indicated by arrow. (E) $Dg^{323}$, muscle 5 attachment site is shifted slightly from its normal position indicated by arrow. (F) $Dg^{323}/Dg^{248}$, muscle 6 is absent, as indicated by arrowheads. All images shown to scale. Scale bar in (A) represents 50μm.

**Figure 2: Sarcomere size is altered in Dg mutants.** Phalloidin stained sarcomeres from muscle 6 (A) yw, (B) $Dg^{248}$, (C) $Dg^{323}$. The graphs (A’-C’) show grey scale intensity across the dashed line drawn on the sarcomeres in A-C. 6 sarcomeres are in the 48μm wide region in yw compared with 7 in the $Dg^{248}$ mutant and 5 in $Dg^{323}$. Red bars indicate one sarcomere. (D) Scatter plot of sarcomere size in the different genotypes where the horizontal line represents the mean value of the data. $Dg^{248}$ sarcomeres are smaller than yw.
but more variable. \(Dg^{323}/Dg^{248}\) and \(Dg^{323}\) show a large variation in sarcomere size and larger sarcomeres compared to \(yw\).

**Figure 3: Sarcomere size changes associated with \(Dg\) RNAi knockdown.** (A) Graph shows gray scale intensity of phalloidin stained sarcomeres shown below. Sarcomeres are aligned on the left of the graph but rapidly become out of phase with each other as the \(tub\)-Gal4::UAS-\(Dg\)-i sarcomeres are smaller than \(yw\) controls. (B) Scatter plot displays sarcomeres size across the different genotypes where the horizontal line represents the mean value of the data. Sarcomere size was measured as the peak to peak distance from graphs as shown in A. Note the increased variance in the 24B-Gal4::UAS-\(Dg\)-i sample compared to controls and the decrease in size of the \(tub\)-Gal4::UAS-\(Dg\)-i sarcomeres. N>100 sarcomeres each genotype.

**Figure 4: Strong knockdown of \(Dg\) in muscles results in large sarcomeres.** (A) Western blot with \(Dg\) antibody detects several bands (marked with arrowheads) in \(yw\) (lane 1) larvae muscle preparation, these bands are reduced in 24B-Gal4::UAS-\(Dg\)-i at 25°C (lane 2) and further reduced when these larvae develop at 30°C (lane 3). Lower panel shows \(\beta\)-tubulin loading control. (B) Sarcomere size of controls and 24B-Gal4::UAS-\(Dg\)-i all at 30°C. 24B-Gal4::UAS-\(Dg\)-i 30°C are consistently larger then controls. N>100 sarcomeres each genotype. *P<0.001. (C) alpha actinin (green) and phalloidin (red) stained sarcomeres. The pattern of alpha actinin and F-actin are normal despite the larger sarcomeres in the 24B-Gal4::UAS-\(Dg\)-i 30°C muscles.
Figure 5: Antibody against Dg reveals staining through the sarcomeres in larval muscles. (A) antibody against the C-terminal Dg epitope reveals staining in larval muscles. Strong Dg puncta are present on the muscle and staining is present at the sarcomeres. Staining is reduced in 24B-Gal4::UAS-Dg-i muscles. (B) An example image of Dg puncta (green) on the muscle surface, the muscle nuclei are stained in red with PI. (C-F) Z-stack of seven 0.6μm sections through sarcomeres. (C) T-tubules marked with Dlg (blue), (D) Dg (green), (E) merge of panels C and D. (F) overlap showing location of F-actin bands of muscle contractile apparatus labeled with phalloidin (red).

Figure 6: Lectin that recognizes N-terminal Dg overlaps Laminin staining in the larval muscles (A, B) Dg antibody (blue), VVA (green) and HRP (red). (A) 24B-Gal4 driven expression of a Dg-C construct leads to strong accumulation of Dg and VVA at post-synaptic regions. (B) 24B-Gal4 driven expression of a truncated Dg, that lacks the extracellular domain, Dg antibody reveals strong accumulation of Dg but VVA staining is not detected. (C, D) Images are single confocal sections, VVA (green), anti-Lam (blue) F-actin (red). (C) yw muscles Lam and VVA overlap at the sarcomeres. (D) 24B-Gal4::UAS-Dg-i 30°C muscle Lam staining appears normal but VVA staining is strongly reduced compared to (C).

Figure 7: rt and tw and double tw;rt mutants show defects in muscle attachment and large sarcomeres. (A) tw larvae showing absence of muscle 5, arrowheads indicated normal location of this muscle. (B) rt mutant showing absence of muscle 5 (arrowheads show normal location). (C) rt/rt muscle 5 (arrowheads) is very thin. (D) tw, muscle 5 (arrowheads) is very thin and attached at an abnormal location. Muscle 4 has split fibers.
(arrow) (E) $tw^1$; $rt^2/rt^2$ muscles 6 is missing (arrowheads). (F) $tw$, $rt$ and $tw;rt$ double mutants all have large sarcomeres compared to $yw$. Images A-E shown to scale. Scale bar in (A) represents 100μm.
Haines et al. Fig. 1

A

B

C

D

E

F

yw

Dg^{248}

Dg^{323}/Dg^{248}

Dg^{248}

Dg^{323}

Dg^{323}/Dg^{248}

8 12 13 6 7

1
Haines et. al. Fig. 3

A

Gray intensity vs. Length (µm)

yw

tub-Gal4::UAS-Dg-i

B

Sarcomere size (µm)

yw, UAS-Dg-i/+ 24B-Gal4/+ 24B-Gal4::UAS-Dg-i tub-Gal4/+ tub-Gal4::UAS-Dg-i 69B-Gal4::UAS-Dg-i
Haines et. al. Fig. 4

A

B

C

yw

UAS-Dg-i/+  24B-Gal4/+  24B-Gal4::UAS-Dg-i

Sarcomere size (μm)

yw

24B-Gal4::UAS-Dg-i 30°C